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MICROMETHOD OF SERUM NEUTRALIZATION OF POLIOMYELITIS.  
USE OF CELLULAR CULTURES ON MOLDED PLASTIC PLATES

[Following is a translation by G. Barski and P. Lepine of the Institute Pasteur, Virological Service, presented to the French Society of Microbiology, session of 1 April 1954, published in the Annales de l'Institut Pasteur.]

The importance of neutralizing antibody research in clinical or undetected poliomyelitis has been demonstrated in innumerable articles dealing with individual diagnoses or epidemiology or immunology of this affection.

The practical interest in a test of serum neutralization whose technique is simple, rapid and sure-fire does not need to be stressed. In fact, only a method which can be applied on a large scale, will allow us to resolve the problem of discovering individuals who do not have neutralizing antibodies, a problem of undeniable importance from the epidemiological viewpoint.

Until recent years it was necessary to use animals for these serum neutralization tests with poliomyelitis virus: monkeys for types I and III and mice for type II [1, 2, 3, 4]. [Translator's Note: The bracketed numerals refer to the bibliographical references listed at the end of this article.] According to Enders, Weller and Robbins [5, 6] it has become possible to use human or simian cells cultivated in vitro in this test. The roller tubes method has been and still is the one used most frequently in practice.

The roller tubes method requires first the preparation of a large number of human or monkey tissue culture tubes, with each tube constituting a test unit. The preparation of these cultures, i.e., obtaining in each tube a sufficiently large cellular growth requires normally seven to ten days, and many maintenance and control operations. During a second step, the serum under test is incubated with three types of virus respectively, and then introduced in the tubes with the cultures.

The method which will be described below reduces the three operations to a single one. The cultivation of detectable cells, the

neutralization tests of the virus with the serum under study, and the placement of the cells into the virus-serum mixture is made at the same time; the results are obtained within 48 hours.

Furthermore, a micro-reaction is involved which proved particularly economical: the volume of serum necessary for this purpose has been reduced to a drop per test, i.e., four drops total, including the proof. The quantity of cells and the volume of the medium have also been reduced to a minimum in the same proportion. Hence, the serum plays a double role: it participates with its antibodies in the neutralization reaction of the virus, and at the same time, by its presence, it completes the medium and there plays an essential role in the growth and existence of the cells.

#### Description of the Method

In order to arrive at a practical accomplishment, it has been necessary to first solve many technical problems: 1. it was necessary to make available, easily and regularly, cellular material of very rapid growth and great sensitivity to the virus; 2. to conceive a culture medium which would assure good growth to the cells and morphological stability (the absence of non-specific degenerescence) in a very small volume and for a sufficiently long time for the specific cytopathogenic effect to be able to be produced in significant quantities; 3. to find the type and material for the equipment with many containers allowing for the execution of these test series and the easy and direct reading of the results.

Cellular Cultures. We have described in a recent article [7] the conditions under which we have obtained re-usable cellular stock starting with muscular subcutaneous tissue obtained by autopsy from new-born infants (primarily among prematures from 6 to 8 months). We recall that the medium utilized (entitled M27) was composed of 20% human placenta serum, 45% cow amniotic liquid, 10% bovine embryonic extract and 25% Hanks solution. [See Note] The composition of this medium was decided upon after very many comparative tests as the most responsive to the demands of very rapid cellular growth, accompanied by a minimum of non-specific degenerescence of the cells. The separation of the primary culture is relatively slow and it is only on the second or third turn that we obtain very many fibroblastic cultures proliferating very rapidly. The re-runs are made every 10 to 15 days with mechanically reduced cellular material in a state of suspension which is poured into a thin plasma layer so as to obtain the most uniform growth possible. These cultures constituted our stock of cellular material for the reaction and it was renewed constantly.

[Note: Amniotic cow liquid prepared according to Enders [8] was provided commercially by the Renard Laboratories, 32 Avenue Henri-Barbusse, Clichy (Seine).]

The cellular material for the test was prepared in the same manner as that used in the re-runs: after sampling and washing in an excess Hanks solution, the entire growth zone is reduced by means of a very sharp instrument to a very fine powder and suspended in a mixture of equal parts of embryonic bovine extract (EEB) and amniotic liquid (LA). For a plate with 25 test units, two to three tubes are sufficient.

Virus. We used for our suspension tests, three stock types of virus: Mahoney (type I), MEF (type II), and Saukett (type III). These suspensions came from cellular cultures in a medium suggested by Enders [8], later called ME, and containing 90% amniotic cow liquid, 5% horse serum and 5% embryonic bovine extract. Following centrifugation for fifteen minutes at 5,000 rpm the suspensions are distributed into ampoules and stored at 75°C. The infection power which varied from  $10^{-4}$  to  $10^{-5}$  was maintained practically unchanged for several months.

Plastic Containers. In order to be able to plan a rapid execution of the test series, it was necessary to replace the culture tubes with grooved honeycombed plates, arranged in rows, with each honeycomb constituting a test unit. To make these plates, we chose plastic araldite B, a polymerized ethoxylic resin [9].

This material has certain special qualities which will be explained in detail in another publication [10]. Let us mention only that araldite B has shown itself free of any toxicity toward live cells which can thus proliferate directly toward the surface which was impossible to attain with plexiglass. Furthermore, araldite has the valuable quality of being able to tolerate, without undergoing any change, sterilization at 100 to 110°C. The molding of this material is relatively easy and we have been able to make plates measuring 120 x 120 mm, 8 mm thick, each containing 25 flat-bottomed honeycombs measuring 10-12 mm in diameter, with distances of 10 mm between them. (See Figure 1)

Araldite is sufficiently translucent to allow an observer to monitor microscopically through its thickness (50 to 100 times enlargement) the cells at the bottom of the honeycomb. Despite the yellow color of the material, the image is clearly satisfactory. The dimensions of the plate (120 x 120 mm) allows us to place it without difficulty upon the microscope slide and examine it across its entire surface.

Measurement. All the constituent materials of the test, the cellular suspension, the serum, the virulent suspension, and the medium are proportioned dropwise with Pasteur pipettes with calibrated spouts. Calibration is accomplished by means of the device described by Lepine and associates, [11]. Each drop contains 38 to 40 mm<sup>3</sup> of liquid.

Test Execution. The operation is started by moistening the bottom of all the honeycombs of the plate using a sterile pad drenched in milksoy plasma; a microdrop of diluted embryonic extract (25%) is added to each. The plate is lightly agitated: a few instances after the plasma coagulation a drop of tissue suspension, prepared in advance, is deposited in each honeycomb. Next, according to an established operational procedure, the serum to be tested is added at the rate of one drop per honeycomb in the horizontal row, and again at the rate of one drop to the three first vertical rows, virulent suspensions of the types I, II and III. The fourth vertical row, reserved as a control row, receives a drop of the ME medium. To complete this and to give the medium a composition approaching that of M27, we add in each honeycomb two drops of a mixture containing 80% of amniotic liquid (LA), 20% of Hanks solution, 200 UC of penicillin and 50 µg of streptomycin per cubic centimeter.

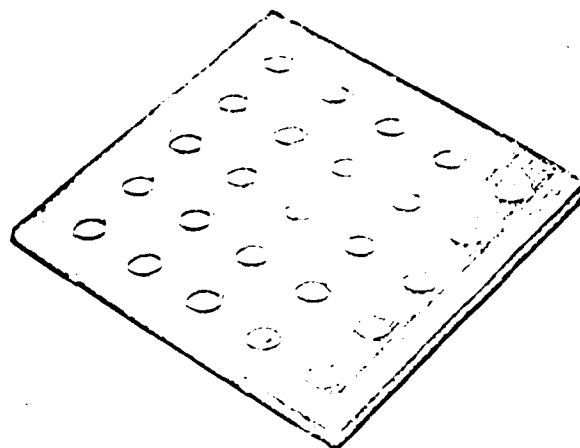


Figure 1. Araldite plate used for the serum neutralization test. The first honeycomb row is covered with an adhesive cellophane tape.

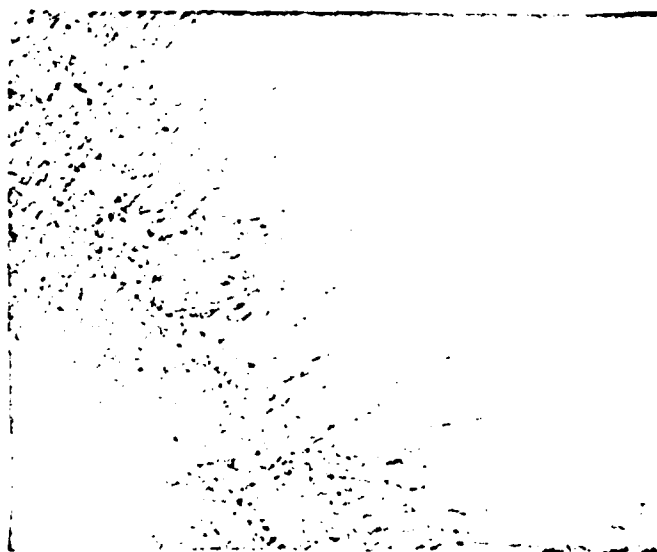


Figure 2. Normal cellular proliferation observed under the microscope (180 x enlargement) at the bottom of a test slide honeycomb in the presence of a serum containing antibodies: no cytopathogenic effect.

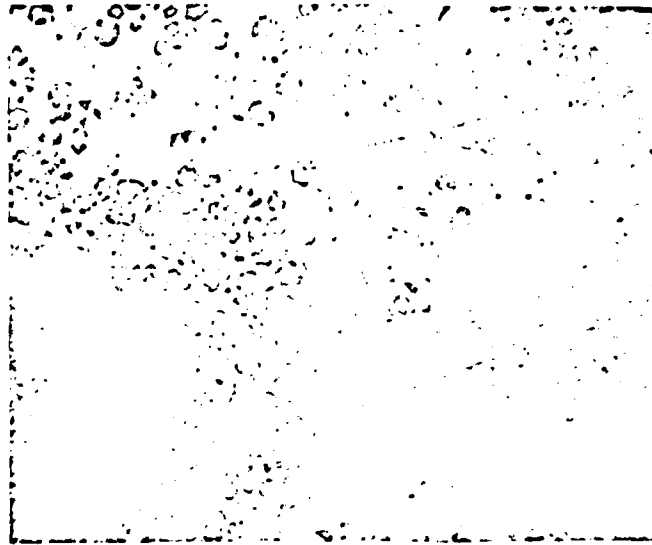


Figure 3. View of the cells (same enlargement as Figure 2) in a honeycomb where the serum does not contain specific antibodies: cellular degeneration due to the cytopathogenic action of the virus.

To cover the honeycombs and isolate them from one another, we used an adhesive cellophane tape over each horizontal row (scotch tape), which adheres very well to the araldite surface, provided it is dry. The contact made sometimes between the honeycomb contents and the adhesive surface does not affect the state of the cells. The adherence of the adhesive to the araldite resists well the oven temperature and the water tightness as well as the separation of the honeycombs during the tests are perfectly well assured. The entire plate is covered with a glass slide of the same dimensions as the araldite plate itself, providing additional safety.

The plates thus prepared are incubated at 37°C and we proceed to the reading of the results after 48 hours.

Results. The cellular suspension used in this test is composed of small cellular agglomerates and it is inevitable that some "blank" plasma fragments do not contain cells. Therefore, the cellular material having been reduced to "dust," the division of the surviving cells is sufficiently uniform for them to be represented in each honeycomb in adequate numbers. Their growth at the bottom of the honeycombs, in a medium which in practice does not differ from M27, is extremely rapid: at the end of 24 hours, it is already considerable, and after 48 hours it generally covers approximately half the honeycomb surface. In the absence of an active virus, the state of the cells is highly satisfactory for four to five days. However, at the end of 48 hours, in those honeycombs where the virus has not been neutralized, signs of its cytopathogenic action are manifest. The migration of the cells starting with fragments has been reduced and the cells present the

characteristic aspect of highly advanced specific degeneration. (See Figures 2 and 3)

We completed, for verification purposes, 72 tests of serum neutralization with human serum, using in part the method described, and in part the roller tube method. The results in each case have been identical.

#### Discussion

Our own observations [10], as well as those of other authors, [11, 12] demonstrated the possibility of obtaining a rapid cytopathogenic effect with highly sensitive cells placed near poliomyelitis virus which can be distinguished at the end of 24 to 48 hours.

It must be stressed that the pre-culture cells, used to in vitro life, always present two major advantages with respect to freshly obtained tissue: 1. they display a very high degree of cellular and growth migration activity which begins practically immediately after being revived; 2. their sensitivity to virus, evaluated according to the final titration point and the rapidity of the appearance of the cytopathogenic effect always becomes higher than that of freshly obtained tissue [7].

The attempt to use cellular suspensions taken directly from the organism and particularly suspensions of artificial ascidian monkey cells yielded frankly negative results in this sense. Suspensions of renal tissue of monkeys or humans have also proved less appropriate for the purpose sought than cells originating from several revived cultures. In turn, the cellular stock He La, isolated by Gey in 1951 from a human carcinoma, and then cultivated in vitro [13] and highly sensitive to the poliomyelitis virus [12], appears to be well suited as cellular material for the method described.

[Note: This stock was forwarded to us by Dr. Charlotte Friend of the Sloan Kettering Institute of New York and by Dr. Audrey Fjelde of the Copenhagen State Serum Institute.]

It is assumed that the fixation of the virus on the cell is practically impossible in the presence of specific neutralizing antibodies. In a recent publication, M. Ledinko and J. L. Melnick [14] demonstrated in particular that as far as poliomyelitis virus in vitro was concerned, the probability of its being fixed on sensitive cells is reduced to a minimum in the particular case where the virus and the serum containing antibodies are introduced simultaneously in the cell culture. We fully confirmed these results and this finding is the basis of our technique.

The fact that at the moment of initiating the test of the cellular material, even in a free state of suspension certainly facilitates largely the interaction between cells and virus, and cells and antibodies. The protection of the cells by specific antibodies occurs apparently very rapidly because in some of the 120 cases of our tests where the antibodies against the given type of virus were present in the serum, the virus did not produce a cytopathogenic effect despite its high concentration.

We also note that by agitating during one hour in the mixing process of the cells, serum, virus and medium, the instant the reaction was started, the test proved useless and we abandoned it.

We cannot presently provide definite quantitative evaluations concerning tolerance limits of our tests. Therefore, it is possible for us to provide some empirically established relationships.

At the moment the serum is placed near the virus it becomes diluted in a 1:5 ratio which corresponds approximately to the serum neutralization conditions used in other methods [14].

The concentration of the virus suspensions employed by us is particularly high. This concentration appears to be useful to obtain the utmost rapidity and completion of the cytopathogenic effect, but it is not impossible that it could be lowered without any ill effects. According to our observations, all the serum tested by this method, and which contains antibodies, precludes the cytopathogenic effect of the corresponding virus very definitely despite the high virus concentration. In all our tests, the classical method of the roller tubes was used as a control test.

Finally, let us add that the same method has given us good results for classifying recently isolated poliomyelitis virus, realized with serum types prepared from monkeys. The monkey serum, in fact, may replace without difficulties the human serum in the honeycombs. In contrast, the horse serum accelerated the appearance of a non-specific degeneration of the cells.

Attempts to carry out serological test series with living tissue infected in vitro with virus were carried out before us by Fulton and Armitage [15]. Therefore, in the Fulton and Armitage method, which in fact constitutes a refinement of the Maitland method adapted to a multiple operation, no true cellular culture is involved, but much more a tissue survival sufficient for the multiplication of the virus. Fulton and his associates did not predict a direct observation of the state of the cells in their cultures, and in order to prove the virus multiplication they took recourse to a second titration operation, for example, using hemagglutination [16].

In contrast, our method, which has the advantage of reducing the entire test of serum neutralization in cellular cultures to a single operation followed by the reading of the results makes use of a true culture which assures to cells not affected by the virus good proliferation conditions. The use of araldite plates permits us to follow their development microscopically without difficulties and without disturbing the test progress. We believe that this device could be usefully employed in other reactions using a cell-virus-antibody system.

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